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Improved Development to Blastocyst of Ovine Nuclear Transfer Embryos Reconstructed during the Presumptive S-Phase of Enucleated Activated Oocytes

K.H.S. CAMPBELL,¹ P. LOI,² P. CAPPAL² and I. WILMUT

AFRC Roslin Institute (Edinburgh), Roslin, Midlothian EH25 9PS, United Kingdom

ABSTRACT

The timing of pronuclear formation and the initiation and duration of the DNA synthetic period (S-phase) were determined during the first cell cycle of electrically activated ovine oocytes matured *in vivo*. Reconstructed embryos were produced by electro-fusion-mediated nuclear transfer of unsynchronized single blastomeres. These were derived from embryos produced *in vivo* at the 16-cell stage (Day 4) and transferred to enucleated metaphase II oocytes at the time of activation or to enucleated activated oocytes during early, mid, and late stages of the presumptive S-phase. The frequency of development to blastocyst was greatest in embryos reconstructed during the presumptive S-phase of enucleated activated oocytes than in embryos reconstructed at the time of activation (mean 55.4% vs. 21.3%). No significant differences were observed when embryos were reconstructed during early, mid, or late stages of the presumptive S-phase (61.3%, 45.7%, and 57.7%, respectively). The results indicate that the use of enucleated activated oocytes as cytoplasts for embryo reconstruction can increase the frequency of development to blastocyst of embryos reconstructed from unsynchronized donor blastomeres.

INTRODUCTION

The reconstruction of early embryos by the transfer of a nucleus from a donor cell (or karyoplast) to an enucleated oocyte or pronuclear zygote (cytoplast) has been accomplished in a variety of mammalian and amphibian species, including mice [1], cattle [2, 3], sheep [4], pig [5], rabbit [6], and amphibians (i.e., *Rana* [7, 8]; *Xenopus* [9]). Although the methods used for embryo reconstruction across these species vary (for review see [10]) they fall broadly into two groups dependent upon the cell cycle stage of the recipient cytoplast. In mammalian species, oocytes arrested at metaphase of the second meiotic division (MII) that have had their chromosomal DNA removed are the cytoplasts most often used. Nuclear transfer is accomplished by the fusion of a donor cell (karyoplast) to the cytoplast using an electrical pulse. The same pulse used to induce fusion also activates the cytoplast. An alternative approach, particularly in mice, is to use enucleated one-cell zygotes as cytoplasts. In this procedure, activation is induced by normal fertilization, and enucleation is then carried out after pronuclear formation. This use of pronuclear zygotes is less common in farm animal species.

After embryo reconstruction, further development is dependent upon a number of factors, two of which are the cell cycle stages of the donor nucleus and recipient cytoplasm at the time of fusion. When MII oocytes are used as cytoplasts, a series of morphological changes is observed in the donor nucleus after fusion, including the induction of nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC), which are then followed by nuclear re-formation (i.e., in cattle [11]). In contrast, when

pronuclear zygotes are used as cytoplasts, no NEBD or PCC have been reported.

The induction of NEBD and PCC is mediated by maturation-promoting factor (MPF), a cytoplasmic activity present in both mitotic and meiotic cells, whose activity increases during G2 and is maximal at metaphase (for review see [12, 13]). In mammalian oocytes the level of MPF activity is high at MII; upon activation the level declines rapidly (i.e., in mice [14]; cattle [11, 15, 16]).

The role(s) of NEBD and PCC in the further development of embryos reconstructed by nuclear transfer is at present unclear. Previous reports have suggested that the induction of NEBD and PCC are essential for the reprogramming of gene expression and that they increase the developmental potential of the reconstructed embryo (e.g., in the rabbit [17]). Studies in this laboratory on DNA synthesis in bovine embryos reconstructed at the time of activation and after the disappearance of MPF activity suggest that an additional explanation for the increased development of embryos reconstructed from MII cytoplasts and G1 karyoplasts is related to DNA replication in the transplanted nucleus during the first cell cycle [11]. Briefly, all nuclei transferred into MII cytoplasts in which MPF activity is high undergo NEBD and PCC. This is followed by nuclear reformation and DNA synthesis regardless of the cell cycle stage of the nucleus. In contrast, when embryos are reconstructed after the disappearance of MPF activity, no NEBD and no PCC are observed. In this situation, G1 and S-phase nuclei initiate or continue DNA synthesis, respectively, whereas no synthesis is observed in transplanted G2 nuclei.

From these experiments we can hypothesize that one possible factor contributing to the development of reconstructed embryos is the maintenance of correct ploidy. If enucleated MII oocytes were used as cytoplasts, then a population of G1 donor nuclei would be required. At the present time, because methods for the synchronization of un-

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¹Correspondence. FAX: (031) 440-0434.

²Current address: Istituto Zootecnico e Caseario 07040, Olmedo, (SS) Italy.

gulate embryos are unreliable, an alternative approach would be to use asynchronous donor nuclei and to control the cell cycle stage of the recipient cytoplasm by activating the enucleated MII oocytes prior to embryo reconstruction [11].

The present study was conducted in order to test this hypothesis by examining the influence of the cytoplasm cell cycle stage on the development of nuclear transplant ovine embryos reconstructed by use of unsynchronized donor blastomeres from 16-cell ovine embryos, which have previously been shown to produce live offspring when transplanted into MII oocytes [18].

MATERIALS AND METHODS

Oocytes and Embryos

Scottish Blackface ewes were synchronized by means of progestagen sponges for 14 days (Veramix, Upjohn, Crawley, UK) and induced to superovulate with single injections of 3.0 mg/day (total 6.0 mg) ovine FSH (Ovagen, Immunochemical Products Ltd., Auckland, New Zealand) on two successive days. Ovulation was induced with an 8-mg single dose of a GnRH analogue (GnRH Receptal, Hoechst, Milton Keynes, UK) 24 h after the second injection of FSH.

Unfertilized MII oocytes were recovered by flushing from the oviduct 31–33 h after GnRH injection, with a phosphate-buffered medium (Ovum Culture Medium [OCM]; Flow Labs, Irvine, Scotland, UK). For the collection of embryos, synchronized ewes were mated to East Friesland tups 24 h after GnRH injection. Embryos were flushed from the uterine horn with OCM at approximately 100 h after GnRH treatment.

Oocyte and Embryo Manipulation

Recovered oocytes were washed in OCM and transferred to medium TCM 199 (Gibco Life Technologies, Paisley, Scotland, UK) containing 10% Fetal Calf Serum (FCS), at 37°C. To remove the chromosomes (enucleation), oocytes were placed in TCM 199 containing 10% FCS, 7.5 µg/ml cytochalasin B (Sigma, Poole, UK), and 5.0 µg/ml Hoechst 33342 (Sigma) at 37°C for 20 min. A small amount of cytoplasm from directly beneath the first polar body was then aspirated by means of a 35-µm pipette (outer diameter). Enucleation was confirmed by exposing the aspirated portion of cytoplasm to UV light and checking for the presence of a metaphase plate. All oocytes were enucleated 34–36 h after GnRH treatment.

Recovered embryos were washed in OCM and then cultured in TCM 199, 10% FCS at 37°C in an atmosphere of 5% CO₂:95% air until use. With the exception of the embryos used as nuclear donors in the 10–12-hpa group (hpa = time of fusion/hours post activation; see *Embryo Reconstruction*), all embryos were used for reconstruction within 3–6 h of recovery from the donor ewe. When storage was necessary, embryos were maintained at room temperature for no more than 6 h.

For nuclear donors, the zona pellucida was cut by a fine glass needle. A single blastomere was then aspirated and introduced inside the zona pellucida of an enucleated oocyte through the slit used for enucleation.

Oocyte Activation

The activation chamber consisted of two parallel platinum electrodes arranged 200 µm apart in a glass Petri dish 9 cm in diameter. Oocytes were placed between the electrodes in 80 µl of activation medium (0.3 M mannitol, 0.1 mM MgSO₄, 0.001 mM CaCl₂ in distilled water [4]). Activation was induced by application of a single DC pulse of 1.25 kV/cm for 80 µs. On each experimental occasion, all oocytes were activated during a 10-min period, 36–37 h after GnRH injection.

Determination of Pronuclear Formation and DNA Synthesis

To assess pronuclear formation, activated oocytes were whole-mounted on ethanol-cleaned glass slides under coverslips attached with a mixture of 5% petroleum jelly:95% wax. Mounted embryos were then fixed for 24 h in freshly prepared methanol: glacial acetic acid (3:1), stained with 45% aceto-orcein, and examined by phase contrast and Direct interference contrast (DIC) microscopy using a Nikon Microphot-SA.

DNA synthesis was measured by incorporation and immunofluorescent detection of the thymidine analogue 5-bromo-2'-deoxyuridine 5'-triphosphate (BrDU, Sigma). Briefly, oocytes, activated oocytes, and embryos were cultured at 37°C, in 5% CO₂ in air, in medium TCM 199 containing 10% FCS and 100 µM BrDU. For pulse-labeling experiments, activated oocytes were transferred to medium containing 100 µM BrDU for 30 min. After incubation, the zona pellucida was removed from the labeled embryos by incubation in 0.5% protease. Zona-free embryos were then washed in PBS, transferred onto coverslips, and allowed to air-dry. Dried embryos were fixed with methanol at –20°C for 20 min, washed in PBS, permeabilized in 0.1% Triton X-100 for 2 min at room temperature, washed in PBS, hydrolyzed in 4 N HCl for 30 min at room temperature, and washed in PBS. Excess PBS was removed, and the coverslips were placed into a humidified chamber. Embryos were then incubated overnight at 4°C in the humidified chamber with 50 µl of primary antibody (rat anti-bromodeoxyuridine; Seralabs, Crawley Down, UK) diluted 1:10 in PBS containing 1% FCS. Coverslips were then washed three times in PBS and incubated with the secondary antibody (fluorescein isothiocyanate-conjugated rabbit anti-rat, Sigma) diluted 1:50 in PBS (containing 1% FCS), for 4 h at room temperature. After incubation, the coverslips were washed three times in PBS, mounted under coverslips on methanol-cleaned glass slides with DABCO (Sigma), and examined by fluorescence microscopy using a Nikon Microphot SA.

Embryo Reconstruction

After determination of the approximate timing of S-phase in activated oocytes, embryos were reconstructed, with enucleated MII cytoplasts and blastomeres from 16-cell embryos used as karyoplasts. Embryos were reconstructed by fusion at the time of activation and also by fusion during early (4–6 hpa), mid (10–12 hpa) and late (16–18 hpa) stages of the presumptive S-phase in activated oocytes. Fusion of manipulated embryos was carried out in the same chamber as oocyte activation with the following changes in protocol. For control (0 hpa) groups, both activation and fusion were induced by application of a single DC pulse of 1.25 kV/cm for 80 μ s in the medium used for activation. Embryos that were reconstructed with enucleated activated oocytes used as cytoplasts (i.e., groups 4–6, 10–12, 16–18 hpa) were induced to fuse by application of three consecutive DC pulses of 0.75 kV/cm for 80 μ s in 0.27 M sucrose. In all cases, the contact surface between the cytoplast and the karyoplast was arranged parallel to the electrodes.

On a single experimental day, all enucleations and manipulations were carried out within 90 min; all cytoplasts and reconstructed couplets (0 hpa) were then activated within a 10-min period.

Embryo Culture and Assessment

Reconstructed embryos were cultured in TCM 199, 10% FCS, 7.5 μ g/ml cytochalasin B at 37°C in 5% CO₂ for 1 h after electropulsing and then transferred to medium without cytochalasin [18]. Fusion was confirmed microscopically, and culture was then continued until all manipulations had been completed. Fused couplets from all groups were then double-embedded in 1% and 1.2% agar (Difco) and transferred to the ligated oviduct of unsynchronized ewes. After 6 days, recipient ewes were killed, and the embryos were retrieved by being flushed from the oviduct with PBS. Embryos were dissected from the agar chips by use of two needles, and development was assessed microscopically. Some embryos that had developed to the blastocyst stage were cultured for 24 h in TCM 199 containing 10% FCS. The number of cells in each embryo was then assessed by fluorescence microscopy after embryos had been incubated in medium containing 5 μ g/ml Hoechst 33342 (Sigma) for 20 min. The number of nuclei in each blastocyst was counted twice, and the mean number was recorded.

Activation of Aspirated MII Karyoplasts

After enucleation, the aspirated karyoplasts containing the metaphase plate were activated by a single electrical pulse as described above. These karyoplasts were returned to culture for 7–10 h and then checked for the presence of a pronucleus by fluorescence microscopy after incubation in Hoechst 33320 for 20 min.

Activation Control

As an activation control, a group of oocytes were manipulated (as described above) without enucleation. After manipulation, the oocytes were returned to culture for 10 h and then examined for the presence of a pronucleus.

Transfer of Blastocysts to Final Recipient Ewes

The estrus cycles of recipient ewes were synchronized by placement of progestagen sponges in the vagina for 14 days. After removal of the sponges, the onset of estrus was designated as Day 0. For development to term, some blastocysts were dissected from the agar chips and transferred to the uterine horn of Day 7 synchronized ewes.

Statistics

All data with the exception of the number of nuclei per blastocyst were analyzed by use of a generalized linear model with extra binomial variance according to McCullagh and Nelder [19]. The number of nuclei per blastocyst was analyzed through a one-way ANOVA. Individual comparisons between means were made by Student's *t*-test.

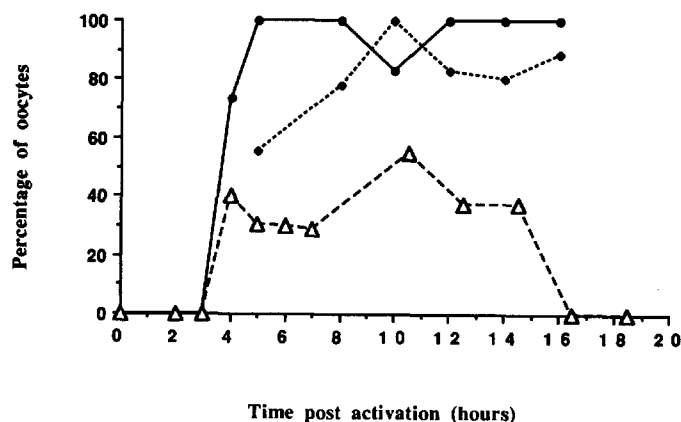


FIG. 1. Graph showing rate of pronuclear formation (solid circles) and the onset and duration of the DNA synthetic period or S-phase in in vivo-matured electrically activated ovine oocytes. Individual groups of oocytes were activated by use of a single DC pulse of 1.25 kV/cm in activation medium (see *Materials and Methods*). Activated oocytes were continuously labeled (solid diamonds), or pulse-labeled (open triangles) with BrDU (see *Materials and Methods*).

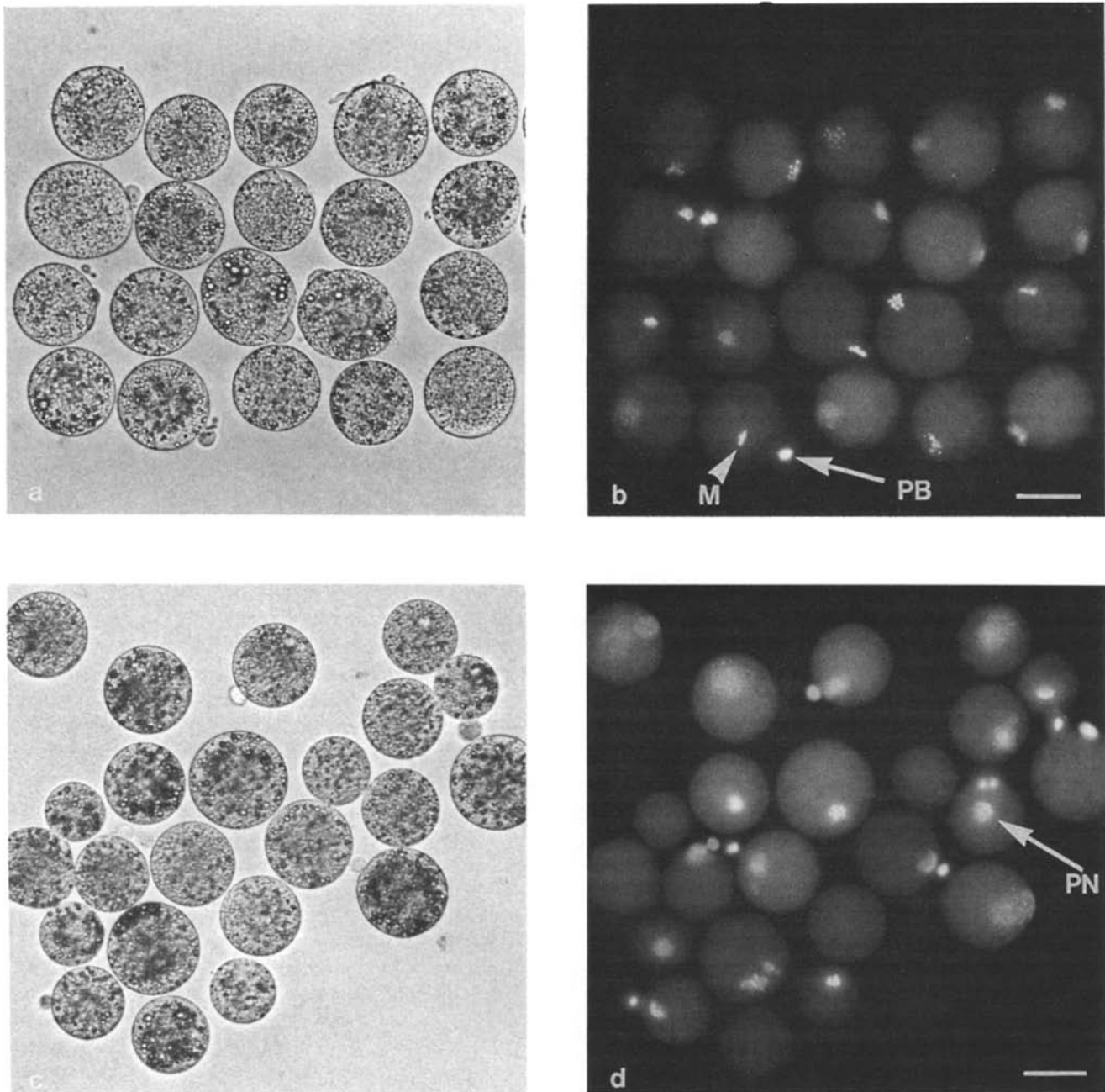


FIG. 2. DIC and fluorescence images of karyoplasts aspirated from in vivo-produced ovine MII oocytes (a and b) and activated karyoplasts 7 h post electropulsing (c and d). The karyoplasts were aspirated, activated and, stained as described in *Materials and Methods*. In some aspirated karyoplasts both the metaphase plate (M) and the first polar body (PB) are visible (b). Panels c and d show a mixed population of karyoplasts with and without pronuclei (PN) and are not indicative of the overall frequency of pronuclear formation. Bar = 20 μm . $\times 200$.

RESULTS

Pronuclear Formation and DNA Synthesis in Unenucleated, Electrically Activated, In Vivo-Matured Ovine Oocytes

In preliminary experiments, the frequency and the rate of pronuclear formation were determined in single batches of oocytes. The onset and duration of S-phase in activated

oocytes were then determined by both pulse and continuous labeling with BrdU. The results from these experiments are summarized in Figure 1. Overall, $86.5 \pm 4.5\%$ (81 of 89) of oocytes formed one or more pronuclei. Pronuclei became visible 3–4 h after activation, and all “activated” oocytes contained pronuclei by 5 h. Within the sample of activated oocytes, DNA synthesis was observed to commence rapidly after pronuclear formation in some oo-

TABLE 1. Influence of cytoplasm cell cycle stage on the fusion of embryo couplets when using unsynchronized blastomeres derived from embryos at the 16-cell stage as karyoplasts.

Treatment hpa*	Number of couplets	Proportion of couplets fusing \pm SE
0	112	0.91 \pm 0.030 ^a
4–6	122	0.70 \pm 0.047 ^b
10–12	143	0.69 \pm 0.043 ^b
16–18	150	0.69 \pm 0.043 ^b

*hpa = time of fusion/hours post activation.

^{a,b} $p < 0.001$.

cytes. However, within the population, the highest frequency of DNA synthesis was not observed until approximately 10–12 h after activation (i.e., 5–7 h after maximal pronuclear formation), suggesting that the period between pronuclear formation and the initiation of DNA synthesis may vary between individual parthenotes. Within the population, DNA synthesis continued for approximately 12 h (i.e., 4–16 h post activation). However, at any time point only 40–50% of parthenotes in the sample incorporated BrDU, suggesting that the duration of S-phase is less than 12 h in individual parthenotes.

As an activation control, 18 oocytes were sham-manipulated (without removing the metaphase plate), returned to culture, and examined for pronuclear formation. Of these oocytes, 22.2% (4 of 18) activated spontaneously and formed a pronucleus.

Activation of MII Karyoplasts

In total, 328 aspirated MII karyoplasts (see Fig. 2, a and b) were electropulsed, and 266 (81.1%) of these formed pronuclei (Fig. 2, c and d). A comparison of the mean frequency of pronuclear formation between batches of oocytes on different experimental days showed that overall 82.0% (SEM \pm 3.41) of aspirated karyoplasts formed pronuclei. No statistical differences were observed in the frequency of activation of karyoplasts from different experimental batches.

TABLE 2. Development during 7 days in temporary recipients of ovine embryos reconstructed by the fusion of single blastomeres, derived from 16-cell embryos (Day 4) to enucleated MII oocytes at the time of activation and during early (4–6 hpa), mid (10–12 hpa) and late (16–18 hpa) stages of the presumptive S-phase.

Treatment hpa*	Number replicates	Number embryos recovered	Proportion (\pm SE) of embryos developing to >1-cell	Proportion (\pm SE) of embryos developing to blastocyst
0	12	75	0.62 \pm 0.065 ^a	0.21 \pm 0.060 ^a
4–6	4	32	0.84 \pm 0.078 ^b	0.61 \pm 0.110 ^f
10–12	7	59	0.78 \pm 0.064 ^c	0.46 \pm 0.082 ^a
16–18	8	78	0.87 \pm 0.044 ^d	0.58 \pm 0.070 ^b

*hpa = time of fusion/hours post activation.

^{a,b} $p < 0.050$, ^{a,c} $p < 0.100$, ^{a,d} $p < 0.005$, ^{a,f} $p < 0.010$, ^{a,g} $p < 0.050$, ^{a,h} $p < 0.001$.

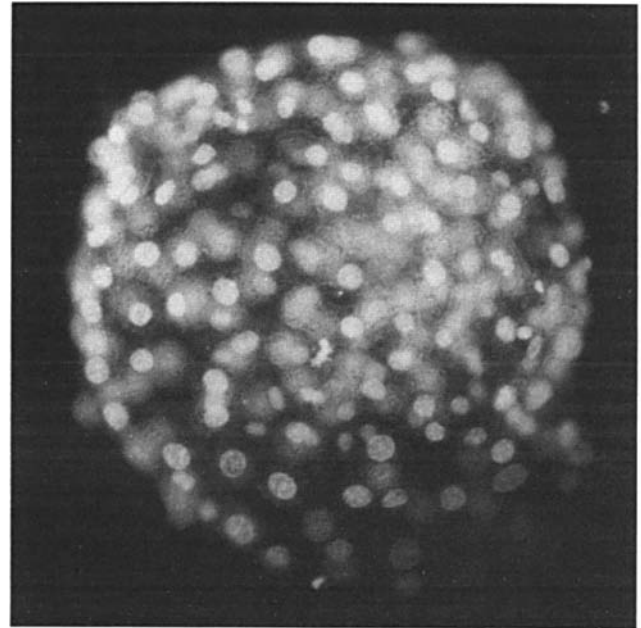


FIG. 3. Hoechst 3342-stained ovine blastocyst obtained on Day 7 after culture of an ovine embryo produced by nuclear transfer in the ligated oviduct of a temporary recipient ewe. $\times 500$.

Electrofusion

After electropulsing, all reconstructed embryos were returned to culture and monitored for fusion. Overall, 74% of manipulated couplets fused (390 of 527). Differences in the rate of fusion between different treatment groups showed a significant decrease in the rate of fusion after activation ($p < 0.001$; Table 1). No significant differences in the rate of fusion were observed between groups reconstructed between 4 and 18 h after activation.

Cell Cycle Stage of Donor Blastomeres

The cell cycle stage of each donor blastomere at the time of embryo reconstruction could not be ascertained. However, immediately after the fusion pulse was applied to reconstructed couplets, residual blastomeres from the disaggregated embryos were pulse-labeled in TCM 199 containing 10% FCS and 100 μ M BrDU for 30 min. Labeled blastomeres were then air-dried onto clean glass slides,

TABLE 3. Mean nuclear number in blastocysts derived from embryos reconstructed as in Table 2.

Treatment hpa*	Number of blastocyst examined	Mean number nuclei \pm SEM
All groups	86	179.06 \pm 6.4
0	14	160.90 \pm 18.1
4–6	14	179.20 \pm 15.9
10–12	23	186.10 \pm 11.6
16–18	35	181.60 \pm 10.1

*hpa = time of fusion/hours post activation.

TABLE 4. Development in vivo of nuclear transfer embryos recovered as blastocysts from temporary recipients and transferred to the uteri of final recipients.

Treatment group hpa*	Number of embryos transferred	Recipient	Transfer days post onset of estrus	Result
0	1	2E118	9	not pregnant
	1	2E226	11	pregnant-live lamb
5-6	1	2E151	7	pregnant-live lamb
	1	2E061	7	not pregnant
	1	2E028	7	"
10-12	2	2E044	7	"
	2	2E261	6	"
16-18	2	2E219	9	"
	2	2E113	9	"
	2	2E240	11	pregnant-live lamb
	2	2E216	11	not pregnant
	2	2E186	11	"

*hpa = time of fusion/hours post activation.

processed as described above, and examined by indirect immunofluorescence microscopy. Overall, 92% of labeled blastomeres ($n = 274$) were in S-phase, and no significant differences were observed between batches.

Development of Ovine Embryos Reconstructed at the Time of Activation and during the Presumptive S-Phase of Enucleated Activated Oocytes

After recovery, the embryos were examined microscopically for development. Results from these experiments are summarized in Table 2. When development to blastocyst was compared, all groups of embryos reconstructed during the presumptive S-phase differed significantly from the control group ($p < 0.05$). No significant differences were observed between groups of embryos reconstructed during early, mid, and late stages of the presumptive S-phase. Some significant differences in the frequency of development past the one-cell stage were observed between embryos reconstructed at the time of activation and during the presumptive S-phase (see Table 2).

The mean number of nuclei in all blastocysts recovered after culture in sheep oviducts (as determined by Hoechst staining; see Fig. 3) was 179 ($\text{SEM} \pm 6.42$). Although we were unable to compare this directly with in vivo-produced blastocysts from the same population of sheep, this figure falls into the range previously reported (138–308) for Day 7–8 blastocysts [20]. The numbers of nuclei in a sample of blastocysts recovered from each treatment group are shown in Table 3. No significant differences were observed between these groups.

A few blastocyst-stage embryos from each treatment group were transferred to the uteri of second recipient ewes, and their individual ability to develop to term was determined. Optimally, blastocysts were transferred to recipients on Day 7 following the return to estrus, however, due to the time of year and limitations in the number of available ewes, the

majority of embryos were transferred to recipients that were not in synchrony (see Table 4). In all, 19 embryos were transferred to 12 recipients and 3 lambs; one each from the control (0 hpa), early (5–6 hpa), and late S-phase (16–18 hpa) reconstructed embryos were born.

DISCUSSION

The present study was carried out to test the hypothesis that the development of embryos reconstructed by nuclear transfer is related to interactions between the donor nucleus and the recipient cytoplasm at the time of fusion and during the first cell cycle after reconstruction. Unactivated MII cytoplasts contain high levels of MPF activity; when they are used as cytoplasts, MPF induces NEBD and PCC in the transferred nucleus regardless of the cell cycle stage of that nucleus (i.e., G1, S, or G2). The effects of MPF may be detrimental to development of the reconstructed embryo for two reasons; (1) the induction of PCC in S-phase nuclei has been reported to induce chromosomal abnormalities [21, 22]; and (2) maintenance of an intact nuclear membrane is essential in the control of DNA replication [11, 23]. All nuclei transferred at the time of activation, when MPF levels are high, undergo NEBD; the nucleus then re-forms, and DNA synthesis is observed.

In contrast, nuclei transferred after the disappearance of MPF activity do not undergo NEBD, and DNA synthesis is observed only in nuclei that were in the G1 or S-phase of the cell cycle at the time of transfer. Thus, in addition to chromosomal damage induced by PCC, nuclei in S-phase or G2 phase when transferred into MII cytoplasts may also undergo uncoordinated DNA synthesis, which may result in incorrect ploidy of the reconstructed embryo. In contrast, the donor nuclei in embryos reconstructed using activated MII oocytes (after the disappearance of MPF activity) as cytoplasts, do not undergo NEBD and PCC and coordinated replication of nuclei in all stages of the cell cycle

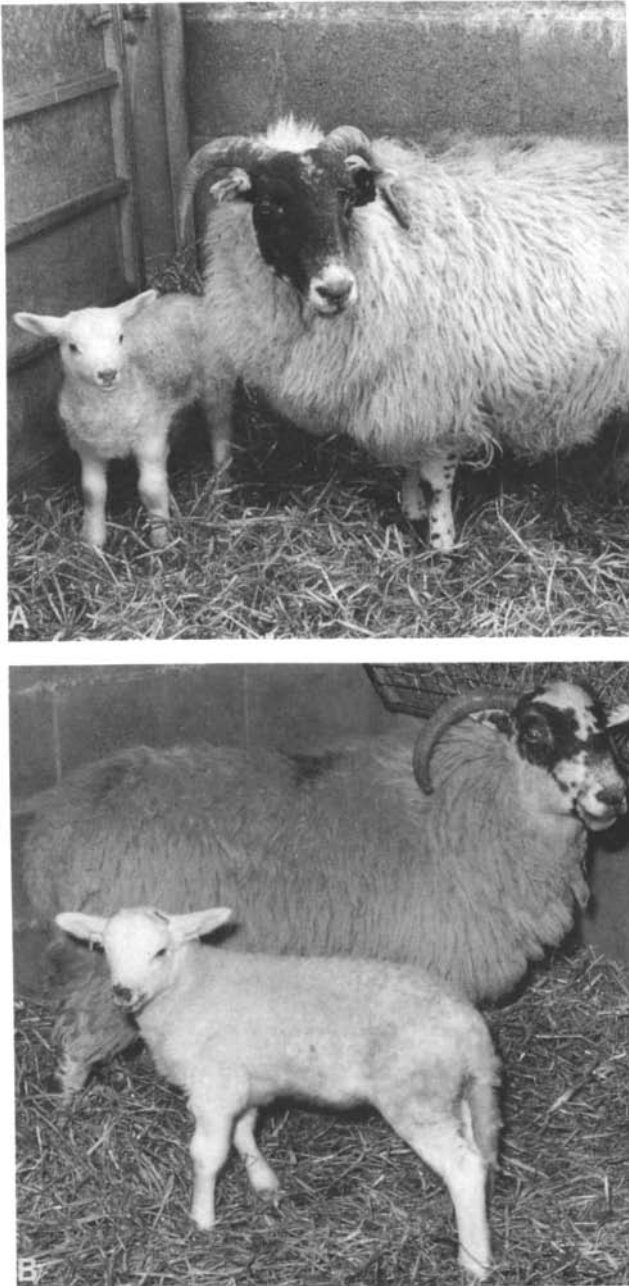


FIG. 4. Lambs born after transfer to final recipients of blastocysts derived from nuclear transfer reconstructed embryos. A) Lamb derived from the control group (0 hpa); B) lamb derived from an embryo reconstructed during the late S-phase (16–18 hpa) of enucleated activated MII oocytes.

occurs. Thus the use of activated oocytes as cytoplasts removes the occurrence of possible chromosomal damage due to PCC and also, during the first cell cycle, coordinates DNA replication of donor nuclei in G1, S- or G2 phases. We have previously referred to such cytoplasts as “The Universal Recipient” [11]. During this study we have not examined the ploidy of reconstructed embryos due to limitations in the amount of biological material; however, two findings support this hypothesis: (1) the increased frequency of devel-

opment to the blastocyst stage of embryos reconstructed using “Universal Recipient” cytoplasts, and (2) a recent report showing that the incidence of chromosomal abnormalities in bovine nuclear transplant embryos is reduced when embryos are reconstructed after the decline of MPF activity in recipient cytoplasts [24]. In addition, we report here that ovine blastocysts that develop from embryos reconstructed from untreated asynchronous 16-cell donor blastomeres using “The Universal Recipient” are capable of development to term and the birth of live offspring.

In these experiments, 92% of control blastomeres were in S-phase at the time of transfer. The hypothesis predicts that the frequency of development of embryos reconstructed at the time of activation will reflect the percentage of blastomeres in G1 at the time of transfer (i.e., < 8%). However, 21% of embryos reconstructed at the time of activation developed to the blastocyst stage. One possible explanation for this higher than expected frequency of development is that after enucleation, but before fusion of the cytoplasm and karyoplast, manipulated oocytes underwent spontaneous activation, and therefore the cell cycle stage of the cytoplasm at the time of fusion was unknown. In a control experiment, 22% of sham enucleated oocytes underwent spontaneous activation. Thus the 21% development to blastocyst observed in the control group (0 hpa) may reflect the proportion of nuclei that were in G1 at the time of transfer plus the proportion of oocytes in which MPF activity had declined by the time of fusion.

Although a significant increase in the frequency of development to the blastocyst stage is reported in this study, this may not reflect the maximum possible frequency of development in reconstructed embryos. Exposure of the donor chromatin to the host cytoplasm by transfer at the time of activation when MPF levels are maximal may further increase the developmental potential of reconstructed embryos. However, from the results reported here and the previously reported replication patterns observed in bovine embryos reconstructed at defined cell cycle stages [11], we have suggested that when cytoplasm MPF levels are high, increased development will be observed only when G1 nuclei are transplanted. This prediction is supported by experiments in both mice [25] and rabbits [22]. In both species, when nuclei at defined cell cycle stages were transplanted into MII cytoplasts at the time of activation, the frequency of development to blastocyst was greater when the donor nuclei were in the early part of the cell cycle (G1) than when nuclei were in the mid-stage (S-phase) of the cell cycle. However, in mice a significant amount of development was also observed when G2 nuclei were transferred [25]. In these experiments, when G2 nuclei were transferred at the time of activation, 67.5% of the reconstructed embryos expelled a polar body after activation. However, only 24.3% of embryos reconstructed from S-phase nuclei and 0% reconstructed from G1 nuclei expelled a polar body. In contrast to the observations in mice, the ex-

trusion of a polar body following embryo reconstruction has not been observed in rabbits [6, 22, 26], sheep [4, 18], or cattle [2, 11, 27]. Thus the observations in mice are also compatible with the hypothesis proposed, in that correct ploidy of embryos reconstructed from G2 nuclei would possibly be maintained after the extrusion of a polar body if segregation of the genetic material was equal and resulted in the formation of a diploid nucleus that was then replicated during the first cell cycle.

A number of alternative explanations may be proposed to explain the results presented here. First, because an electric pulse was used to induce both activation of the cytoplasm and karyoplast/cytoplasm fusion, those embryos reconstructed during the presumptive S-phase received an additional activation stimulus. In these groups, the fusion medium consisted of sucrose alone, and therefore no influx of external calcium ions would be expected; however, we cannot exclude the release of calcium ions from internal sources in response to the fusion pulse. Second, the increased development observed is due to the synchronous transfer of S-phase blastomere nuclei into S-phase cytoplasts. From the observations made, we are unable to define the exact cell cycle stage of each individual cytoplasm or to define precisely whether donor blastomeres were in early, mid, or late S-phase at the time of transfer. We suggest that the major factor influencing development is the maintenance of an intact nuclear membrane by transfer of donor nuclei after the disappearance of MPF. No measurements of MPF activity were made; however, by definition, MPF activity declines prior to pronuclear formation, and therefore we suggest that in all reconstructions, with the exception of the 0-hpa control group, MPF activity had declined. No measurements of DNA synthesis were made in the reconstructed embryos; we thus are unable to confirm that DNA replication was either continued or completed in the transferred nucleus. Experiments in reconstructed bovine embryos have shown that replication is initiated or continued when G1 or S-phase nuclei are transferred into enucleated activated cytoplasts after the disappearance of MPF activity [11]. As yet no experiments confirm that replication is completed in such nuclei; however, experiments in mice have demonstrated that the first cell cycle is elongated when nuclei in early S-phase are transferred to cytoplasts in late first cell cycle. These observations suggest that in mice, at least, a control mechanism is operating which links the completion of S-phase to the onset of mitosis [28]. In order to further investigate the interactions and developmental potential of embryos reconstructed from defined cell cycle stages, reliable methods for the synchronization of embryos or embryonic stem cell populations are required.

A significant difference was observed between the rate of fusion of the control (0 hpa) group and those embryos reconstructed during the presumptive S-phase. Although different media and different electrical pulses were em-

ployed in these groups, other reports have suggested that the cell cycle stage of both the donor cells and the recipient cytoplasm may influence the frequency of fusion [25]. At present we are unable to confirm that the cell cycle stage of either the donor cell or the recipient cytoplasm can influence fusion.

The primary aim of this trial was to compare development to the blastocyst stage. During the latter stages of the experiment, we transferred some blastocysts to recipient ewes. Of 19 embryos transferred to 12 recipients, 3 pregnancies were induced, which resulted in the birth of 3 live offspring, one each from the control (0 hpa), early (5–6 hpa), and late (16–18 hpa) S-phase groups. Thus 15.7% of the transferred embryos developed to term. Although we report these births, for several reasons we believe that the frequency of development does not truly reflect the developmental potential of each group. First, although attempts were made to synchronize the onset of estrus of the recipients with the stage of the embryo at the time of transfer, because of limited numbers of animals and also time of year, many of the embryos were transferred asynchronously (see Table 4). Second, no Day 7 *in vivo*-produced blastocysts were simultaneously transferred from donor to recipient ewes as a control of the frequency of induction of pregnancy.

The use of enucleated activated MII oocytes as recipients for embryo reconstruction mimics the use of enucleated pronuclear zygotes as recipient cytoplasts. However, the use of pronuclear zygotes removes the necessity to activate recipient oocytes. Experiments in both cattle [29] and pigs [30] have shown that visualization of the pronuclei by centrifugation does not affect the developmental potential of one-cell zygotes. Similarly, when pronuclear exchange is carried out in both bovine or porcine zygotes, the reconstructed embryos are capable of development [3, 5]. However, experiments in cows using donor blastomeres from 2-, 4-, and 8-cell embryos resulted in no development [3]. The lack of development observed in ungulate embryos reconstructed from enucleated zygotes suggests that other as yet unidentified factors are required for the development of reconstructed embryos. One possibility is that these factors are associated with the pronuclei and are therefore removed by enucleation of zygotes.

During this study, 82.0% (± 3.41) of aspirated karyoplasts became activated, 83.0% of embryos reconstructed during the presumptive S-phase cleaved once, and 55.0% developed to the blastocyst stage. These figures suggest that the rate of cleavage of embryos reconstructed during S-phase is equivalent to the frequency of activation. If we extrapolate further, then the true frequency of development to blastocyst of fused and activated couplets is 66.7% (± 7.7) overall for embryos reconstructed during S-phase or 74.0%, 56.0%, and 70.0% of embryos reconstructed during early, mid, and late stages, respectively. In contrast, when embryos were reconstructed from MII cytoplasts, then only

62.0% (75.6% of activated couplets) of embryos cleaved once, suggesting that completion of the first cell cycle was restricted in this combination.

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